Effect of artemisinin derivatives on apoptosis and cell cycle in prostate cancer cells

Colm Morrissey[a], Byron Gallis[b], Jeffrey W. Solazzi[a], Byung Ju Kim[c], Roman Gulati[e], Funda Vakar-Lopez[d], David R. Goodlett[b], Robert L. Vessella[a,f] and Tomikazu Sasaki[c]

Artemisinin is a plant-derived anti-malarial drug that has relatively low toxicity in humans and is activated by heme and/or intracellular iron leading to intracellular free radical formation. Interestingly, artemisinin has displayed anti-cancer activity, with artemisinin dimers being more potent than monomeric artemisinin. Intracellular iron uptake is regulated by the transferrin receptor (TfR), and the activity of artemisinin depends on the availability of iron. We examined the level of TfR in prostate cancer (PCa) tumor cells, synthesized two new artemisinin dimers, and evaluated the effect of dihydroartemisinin and artemisinin dimers, ON-2Py and 2Py, on proliferation and apoptosis in PCa cells. TfR was expressed in the majority of PCa bone and soft tissue metastases, all 24 LuCaP PCa xenografts, and PCa cell lines. After treatment with dihydroartemisinin, ON-2Py, or 2Py all PCa cell lines displayed dose-dependent decrease in cell number. 2Py was most effective in decreasing cell number. An increase in apoptotic events and growth arrest was observed in the C4-2 and LNCaP cell lines. Growth arrest was observed in PC-3 cells, but no significant change was observed in DU 145 cells. Treatment with 2Py resulted in a loss of the anti-apoptotic protein survivin in all four cell lines. 2Py treatment also decreased androgen receptor and prostate-specific antigen expression in C4-2 and LNCaP cells, with a concomitant loss of cell cycle regulatory proteins cyclin D1 and c-Myc. This study shows the potential use of artemisinin derivatives as therapeutic candidates for PCa and warrants the initiation of preclinical studies. *Anti-Cancer Drugs* 21:423–432 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Departments of [a]Urology, [b]Medicinal Chemistry, [c]Chemistry, [d]Pathology, University of Washington, [e]Fred Hutchinson Cancer Research Center and [f]Department of Veterans Affairs VA Medical Center, Seattle, Washington, USA

Correspondence to Dr Colm Morrissey, PhD, Genitourinary Cancer Research Laboratory, Department of Urology, PO Box 356510, University of Washington, Seattle, WA 98195, USA
Tel: +1 206 543 1461; fax: +1 206 543 1146;
e-mail: cmorriss@u.washington.edu

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Introduction

The American Cancer Society estimates that during 2008 approximately 28 660 men died of metastatic prostate cancer (PCa) in the United States. While chemotherapeutic strategies show some promise, there is no effective therapy that substantially prolongs survival for hormone-refractory PCa. A number of studies have suggested that artemisinin may be useful in the treatment of solid tumors including PCa [1–4].

Artemisinin has been used as an anti-malarial drug for a number of years now [3,5,6]. It has a defined mechanism of action, with artemisinin and its derivatives effecting heme-mediated decomposition of the endoperoxide bridge in artemisinin to produce carbon-centered free radicals [7]. This heme-catalyzed excess of reactive oxygen species induces apoptosis through a mitochondrial-mediated pathway, ER stress induction, or blocking cell cycle kinetics [2,8,9]. These cellular effects, in combination with relatively low toxicity in humans [3], make artemsinin an attractive candidate drug for the treatment of solid tumors.

The levels of transferrin receptor (TfR) can determine the intracellular levels of holo-transferrin [Fe(III)], the iron (III) transport protein in the blood that plays an important role in the electrocatalytic reduction of artemisinin, which can catalyze the cleavage of the endoperoxide bridge in artemisinin [10]. Therefore, we set out to determine the expression of TfR in human PCa metastases, xenografts and cell lines. Furthermore, artemisinin dimers have shown potent anti-cancer activities both *in vitro* and *in vivo* [3, 11–13], and a relatively small number of articles have been published on the effectiveness of artemisinin and its derivatives in inhibiting the growth of PCa cells *in vitro* and *in vivo* [1–3,8].

Therefore, we synthesized two artemisinin dimers (2Py-ON and 2Py) and tested their ability to induce apoptosis and/or proliferation in PCa cell lines *in vitro*. In summary, the aim of this study was to determine the levels of TfR in PCa and to elucidate the effects of artemisinin, 2Py-ON, and 2Py on growth inhibition and apoptosis in C4-2, DU 145, LNCaP, and PC-3 cells *in vitro*.

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Materials and methods

Preparation of 2Py

Preparation of dimer hydrazide (dimer-$NHHH_2$): The trioxane dimer acid (500 mg, 0.8 mmol, prepared using Posner's method [3]) was treated with tetrafluorophenol (412 mg, 2.56 mmol) in the presence of EDCI-HCl [3-(N,N′-dimethylaminopropyl)ethyl carbodiimide hydrochloride; 416 mg, 1.6 mmol] and triethylamine (0.5 ml, 4 mmol) in dichloromethane (16 ml) at room temperature. After stirring overnight, anhydrous hydrazine (94 µl, 3.2 mmol) in dry DMF (0.5 ml) was added. The solution was then stirred for a further 1 h. The reaction was quenched with water and extracted with chloroform. The organic layer was dried over magnesium sulfate, filtered, and concentrated. The residue was purified on silica gel with dichloromethane:methanol (40:1) and recrystallized in methanol. Isolated yield, 348 mg (68%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.97 (bs, 1H), 5.30 (s, 1H), 5.17 (s, 1H), 4.16 (dd, J = 10.5, 6.5 Hz, 1H), 4.10 (dd, J = 11.0, 6.5 Hz, 1H), 3.70 (bs, 1H), 2.75 (m, 1H), 2.56 (m, 1H), 2.50–2.42 (m, 1H), 2.32 (td, J = 14.0, 3.5 Hz, 2H), 2.21 (m, 1H), 2.07–1.97 (m, 2H), 1.97–1.89 (m, 1H), 1.89–1.72 (m, 4H), 1.68–1.56 (m, 4H), 1.55–1.33 (m, 12H, including two singlets at δ 1.44 and 1.40), 1.32–1.18 (m, 4H), 0.96 (d, J = 6.0 Hz, 3H), 0.94 (d, J = 6.5 Hz, 3H), 0.85 (d, J = 7.5 Hz, 3H), 0.83 (d, J = 7.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.8, 103.8, 103.3, 89.6, 88.3, 81.4, 81.1, 71.9, 52.7, 52.2, 44.9, 44.3, 42.8, 37.7, 37.4, 36.9, 36.6, 34.7, 34.5, 34.0, 33.1, 30.3, 30.2, 26.3, 26.2, 25.0, 24.9, 24.7, 20.4, 20.3, 13.7, 12.9; LRMS (ESI), m/z $[M + H]^+$ 635.4.

To a solution of trioxane dimer hydrazide (50 mg, 0.078 mmol) in methanol (1 ml), pyridine-2-carboaldehyde (7.5 µl, 0.08 mmol) at 20–25°C was added. After stirring for 4 h to overnight, the reaction mixture was filtered or chromatographed and then recrystallized. Dimer-2Py; isolated yield; 54 mg, 96% as an amorphous solid (E:Z = 4.5:1): ^{1}H NMR (500 MHz, CD_3OD) δ 8.54 (d, J = 5.0 Hz, 2H), 8.27 (d, J = 8.0 Hz, 1H), 8.16 (s, 1H), 7.88 (dd, J = 7.5, 6.5 Hz, 2H), 7.41 (dd, J = 5.5, 5.0 Hz, 2H), 5.45 (s, 1H), 5.27 (s, 1H), 4.16–4.23 (m, 1H), 4.11–4.05 (m, 1H), 2.80 (m, 1H), 2.69 (m, 1H), 2.63 (m, 1H), 2.30–2.13 (m, 3H), 2.07–1.77 (m, 8H), 1.73–1.63 (m, 3H), 1.60–1.15 (m including singlets at δ 1.31 and 1.19, 16H), 1.02–0.85 (m, 14H); ^{1}H NMR (500 MHz, CD_3OD, *Z*) δ 8.19 (d, J = 8.0 Hz), 7.01 (s), 5.43 (s), 5.36 (s), 1.37 (s), 1.23 (s); ^{13}C NMR (125 MHz, CD_3OD) δ 176.2, 154.8, 150.1, 148.4, 138.7, 126.0, 122.4, 104.78, 104.76, 90.5, 89.9, 82.5, 82.3, 76.9, 74.8, 54.2, 54.1, 46.3, 46.1, 43.4, 38.7, 38.5, 37.7, 37.6, 35.9, 35.8, 34.5, 33.6, 31.7, 26.4, 26.37, 26.1, 26.0, 25.9, 20.8, 20.7, 13.8, 13.6; LRMS (ESI), m/z $[M + H]^+$ 724.7.

Preparation of dimer ON-2Py

To a mixture of dimer alcohol (500 mg, 0.8 mmol, prepared by Posner's method [12]), triphenylphosphine (1.73 g, 3.2 mmol), *N*-hydroxyphthalimide in chloroform (12 ml) a solution of DEAD (diethyl azidocarboxylate, 0.52 ml, 3.2 mmol) in chloroform (5 ml) at room temperature was slowly added. The mixture was stirred overnight and quenched with water. The solution was extracted with chloroform, dried over magnesium sulfate, and concentrated. The residue was purified on silica gel with hexanes (EtOAc; 4 : 1 to 3 : 1) to give the corresponding product. Isolated yield, 446 mg (74%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.85–7.78 (m, 2H), 7.76–7.69 (m, 2H), 5.40 (s, 1H), 5.34 (s, 1H), 4.50–4.18 (m, 5H), 2.75 (q, J = 6.6 Hz, 1H), 2.65 (q, J = 6.6 Hz, 1H), 2.42–2.22 (m, 3H), 2.22–2.08 (m, 1H), 2.04–1.15 (m, 16H including singlet at δ 1.37), 0.96 (dd, J = 6.0, 3.6 Hz, 6H), 0.91 (dd, J = 7.5, 2.1 Hz, 6H). The dimer, phthalimide (202 mg, 0.27 mmol), was treated with hydrazine monohydrate (40 µl, 0.81 mmol) in ethanol (7 ml) at room temperature. After 1 h, the reaction mixture was filtered. After concentration, the residue was purified on silica gel by eluting with dichloromethane methanol (45 : 1) to give the corresponding aminoxy dimer in 98% yield (189 mg); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.38 (s, 1H), 5.34 (s, 1H), 5.31(s, 1H), 4.36 (m, 1H), 4.16 (m, 1H), 3.97 (d, J = 4.6 Hz, 2H), 2.79–2.55 (m, 2H), 2.33(t, J = 14.0 Hz, 2H), 2.20–1.30 (m, 19H including singlet at δ 1.41), 0.96 (d, J = 6.0 Hz, 6H), 0.86 (dd, J = 7.2, 2.1 Hz, 6H); LRMS (ESI), m/z $[M + H]^+$ 622.5. To a solution of aminoxy dimer, artemisinin (50 mg, 0.08 mmol), in methanol (2 ml) pyridine-2-carboaldehyde (8 µl, 0.08 mmol) was added at room temperature. After stirring for 4 h to overnight, the solvent was removed then purified on silica gel with hexanes (EtOAc; 4 : 1). Isolated yield, 47 mg (83%); ^{1}H NMR (500 MHz, CD_3OD) δ 8.54 (d, J = 5.0 Hz, 1H), 8.15 (s, 1H), 7.92 (d, J = 7.5 Hz, 1H), 7.85 (td, J = 7.5, 1.5 Hz, 1H), 7.40 (ddd, J = 7.5, 5.0, 1.0 Hz, 1H), 5.49 (s, 1H), 5.33 (s, 1H), 4.45–4.32 (m, 3H), 4.24 (m, 1H), 2.66 (q, J = 7.5 Hz, 1H), 2.57 (q, J = 7.5 Hz, 1H), 2.40–2.20 (m, 3H), 2.10–1.73 (m, 8H) 1.72–1.11 (m, 19H including two singlets at δ 1.37 and 1.33), 0.96 (d, J = 6.0 Hz, 3H), 0.92–0.86 (m, 9H); ^{13}C NMR (125 MHz, CD_3OD) δ 153.1, 150.4, 149.3, 138.7, 125.8, 122.3, 104.8, 104.4, 90.8, 90.2, 82.5, 82.45, 78.6, 75.4, 73.9, 54.1, 53.9, 46.2, 45.9, 38.7, 38.6, 37.8, 37.7, 36.2, 35.8, 32.2, 32.0, 31.1, 30.9, 26.4, 26.2, 26.1, 26.0, 25.9, 20.1, 13.8, 13.4.

Immunohistochemistry

To assess TfR expression in PCa metastases, human tissue microarrays of formalin-fixed, paraffin-embedded tissues from 22 rapid autopsy patients were used for immunohistochemical analyses [14]. To assess TfR expression in 24 LuCaP PCa xenografts (developed at the University of Washington), animals were implanted with each of the LuCaP PCa xenograft lines subcutaneously, and the resultant tumors were formalin fixed and embedded in paraffin as described earlier [15,16]. Five-micron sections of the human tissue microarrays and

subcutaneous LuCaP tumors were deparaffinized, and antigen retrieval was performed in 10 mmol/l of citrate buffer (pH 6) at 120°C. The sections were then incubated with 3% H_2O_2, blocked with avidin/biotin blocking solution (Vector Laboratories Inc., Burlingame, California, USA) and incubated in a 5% chicken/goat/horse serum solution. The sections were stained with 5 μg/ml of mouse anti-human TfR antibody (Zymed Laboratories Inc., South San Francisco, California, USA). Negative control slides were incubated with rabbit IgG (Vector Laboratories Inc.) or mouse anti-MOPC21 (generated in-house from a hybridoma obtained from ATCC, Manassas, Virginia, USA) at the same concentration as the primary antibody. All slides were then incubated with horse anti-mouse biotinylated secondary antibody (1 : 150) (Vector Laboratories Inc.) and developed using the Vectastain ABC kit (Vector Laboratories Inc.) and stable DAB (Invitrogen Corporation, Carlsbad, California, USA), counterstained with hematoxylin, and dehydrated and mounted with Cytoseal XYL (Richard Allan Scientific, Kalamazoo, Michigan, USA). Immunostaining was assessed using the following 4-point categorical compositional scale: 0 = no staining, 1 = faint/equivocal or focal staining, 2 = definite staining of a minority of cells, and 3 = definite staining of a majority of cells. The immunostaining results were determined by consensus by C.M. and F.V.L. (listed authors). Statistical analysis of immunohistochemical comparing bone, liver, and lymph node metastases on tissue microarrays was described earlier [14].

Cell culture and reagents

PC-3, LNCaP, C4-2, and DU 145 cells were maintained in RPMI 1640 with L-glutamine (Invitrogen Corporation) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, Georgia, USA). Dihydroartemisinin (DHA) was obtained from Holleypharma Inc. (Beijing, China). Human holo-transferrin was obtained from Fortune Biologicals Inc. (Gaithersburg, Maryland, USA) (Fig. 1).

Immunocytochemistry

LNCaP and C4-2 cells were cultured on Lab-Tek Chamber slides (Nalge Nunc Naperville, Illinois, USA). After treatment in RPMI 1640 with 10% FBS (Atlanta Biologicals) with dimethylsulfoxide or 15 μmol/l of 2Py for 48 h, the cells were fixed and incubated with rabbit polyclonal anti-human β-catenin antibody (5 μg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) or control rabbit IgG (5 μg/ml; Vector laboratories Inc.) and then with goat-anti-rabbit Alexa Fluor 488 (Molecular Probes Inc., Eugene, Oregon, USA) at a dilution of 1 : 400, respectively, and mounted with ProlongGold anti-fade reagent w/DAPI (Invitrogen Corporation).

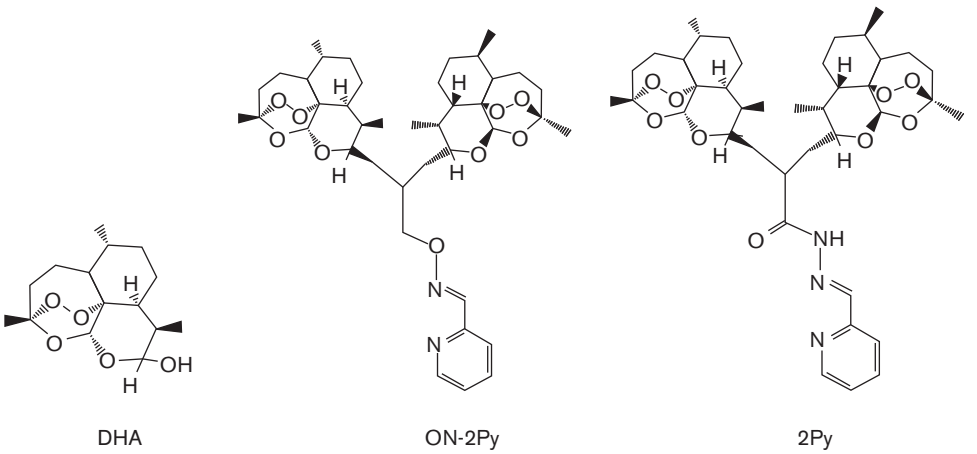
Crystal violet assay

PC-3, LNCaP, and C4-2 cells were grown in 24-well plates to a 50% confluence in RPMI 1640 with 10% FBS and treated with DHA, 2Py, or ON-2Py for 24, 48, or 72 h. Cells were fixed in 10% buffered formalin, washed with PBS, incubated with 1% crystal violet (Sigma-Aldrich, St Louis, Missouri, USA), solubilized with 1% Triton X-100 and analyzed for absorbance at 590 nm on a Tecan GENios Plus (Tecan Group Ltd, Zurich, Switzerland).

MTT assay

PC-3, C4-2, LNCaP, and DU 145 cells were grown in 24-well plates to a 50% confluence in RPMI 1640 with 10% FBS and treated with 2Py (controls were treated with vehicle alone) and/or 12.4 μmol/l of iron-saturated human holo-transferrin for 48 h. The cells were then incubated with MTT, formazan crystals were

Fig. 1



Structures of dihydroartemisinin (DHA), ON-2Py, and 2Py.

solubilized in dimethylsulfoxide and absorbance was measured at 590 nmol/l on a Tecan GENios Plus (Tecan Group Ltd).

Western blot analysis

Whole-cell lysates were prepared as described earlier [17]. Protein levels were determined using the Bio-Rad DC Protein Assay kit (BioRad Laboratories, Hercules, California, USA). Western blotting was performed as described earlier [2]. Antibodies to β-catenin, c-Myc, cyclin D1, androgen receptor (AR), and prostate-specific antigen (PSA) were from Epitomics (Burlingame, California, USA). Antibodies to survivin, bcl-2, and bax were from Cell Signaling Technology (Danvers, Massachusetts, USA), to the transferrin receptor (clone H68.4) were from Zymed (Grand Island, New York, USA), and to β-actin were from Sigma (St Louis, Missouri, USA).

Apoptosis and viability assays

Apoptotic events were described as a percentage of total events with hypodiploid DNA assessed by propidium iodide (PI) incorporation. The cells were harvested by trypsinization, permeabilized with a hypotonic fluorochrome solution (50 μg/ml PI, 3.4 mmol/l sodium citrate, 1 mmol/l Tris, 0.1 mmol/l EDTA, and 0.1% Triton X-100) and incubated on ice for 10 min before analysis. Viability assays were performed on the same samples. The cells were harvested by trypsinization, permeabilized with a hypotonic fluorochrome solution (50 μg/ml PI, 3.4 mmol/l sodium citrate, 1 mmol/l Tris, 0.1 mmol/l EDTA) and incubated on ice for 5 min before analysis. The samples were run on a BD FACScan (BD Biosciences, San Jose, California, USA). Five thousand events were gated on PI intensity and analyzed using Cell Quest software (BD Biosciences).

Statistical analysis

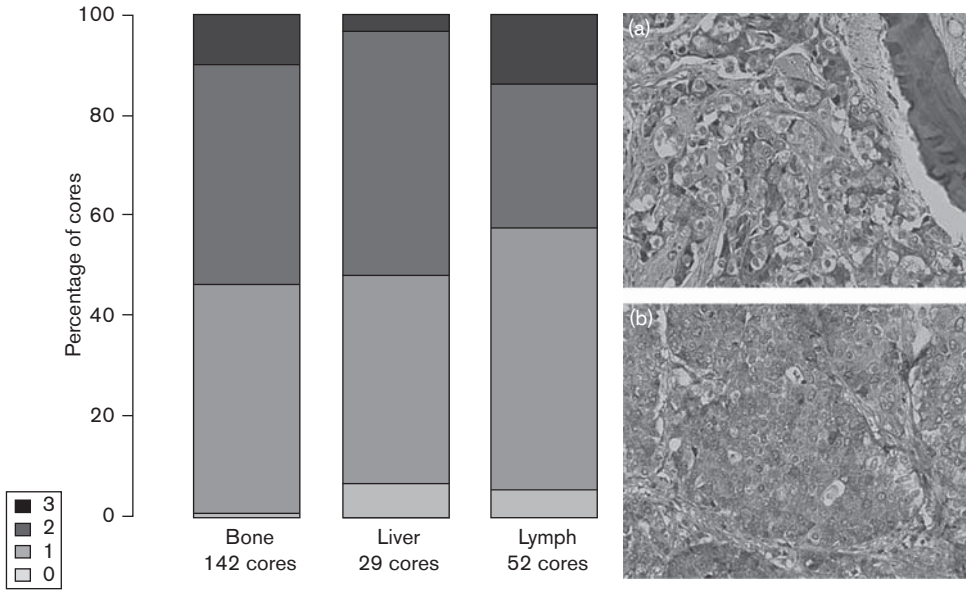
For the in-vitro experiments, the significance of differences was evaluated using the paired Student's *t* tests as appropriate, with *P* values equal to or less than 0.05 indicating statistical significance.

Results

Transferrin receptor expression in PCa metastases, xenografts and cell lines

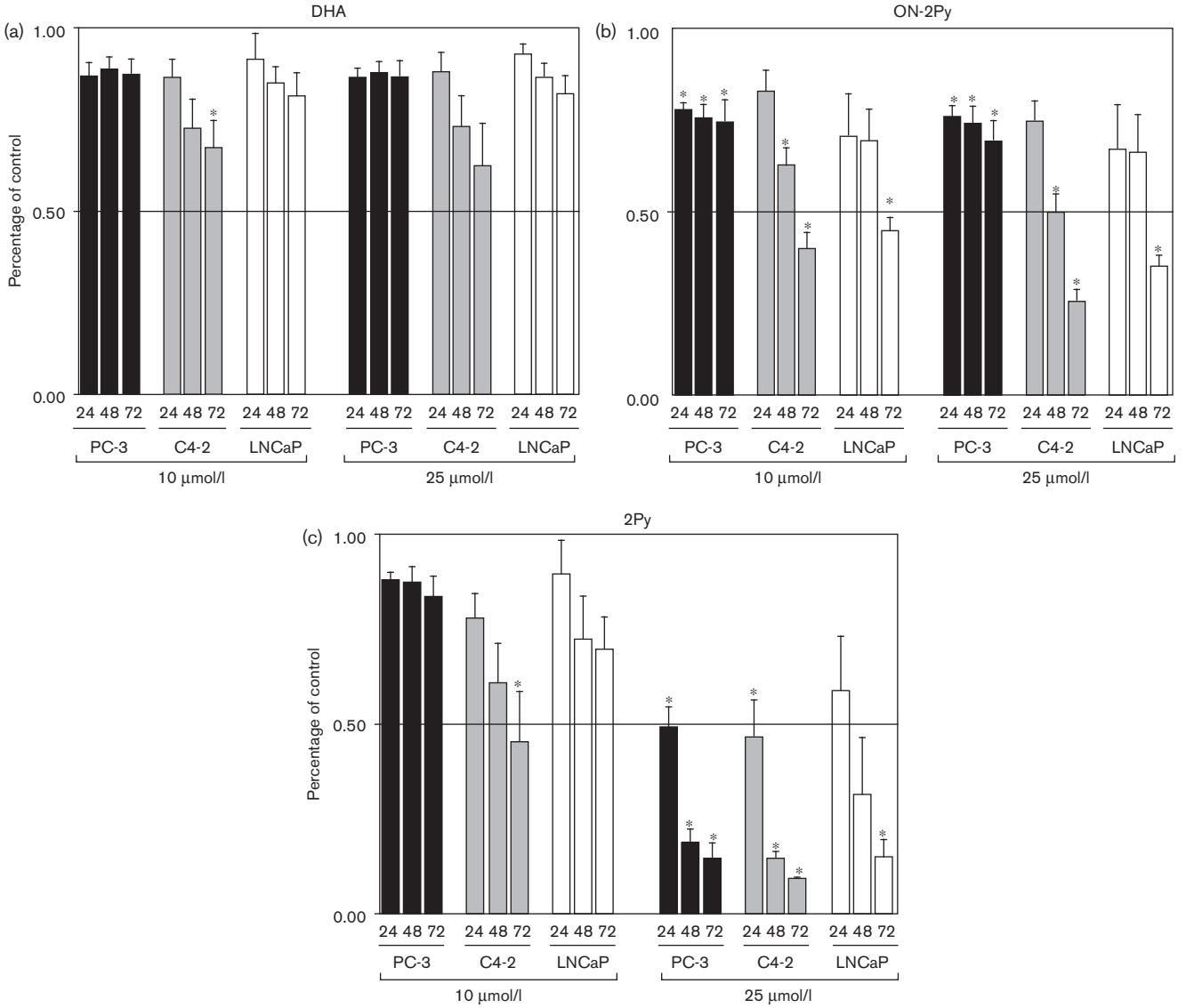
The activity of artemisinin depends on the availability of iron, and intracellular iron uptake depends on the presence of TfR. Therefore, we examined TfR expression in PCa metastases, xenografts, and cell lines. We observed no significant difference in TfR protein expression among PCa bone, liver, and lymph node metastases by immunohistochemical analysis. In PCa bone, liver, and lymph node metastases the expression pattern of TfR was cytoplasmic, with the majority of tumor cells expressing TfR. Intense staining was only observed in a minority of cases (Fig. 2). Cytoplasmic TfR expression was also observed in all 24 PCa LuCaP xenografts and in the C4-2, DU 145, LNCaP, and PC-3 cell lines by immunohistochemistry (data not shown). TfR was also observed in C4-2, DU 145, LNCaP, and PC-3 cells by western analysis with elevated levels in the DU 145 and PC-3 cell lines (data not shown).

Fig. 2



Immunohistochemical analysis of transferrin receptor (TfR) expression in human PCa bone (a) and lymph node (b) metastases from 22 patients (200-fold magnification). There was no evidence that TfR staining intensity varies between bone and soft tissue metastases. Specific immunostaining was assessed on a 4-point scale: 3=intense, 2=defined, 1=faint, and 0=absent.

Fig. 3



Cell number as assessed by crystal violet assay in LNCaP, C4-2, and PC-3 cells. Cells were brought to 50% confluence and then cultured in RPMI 1640 with 10% fetal bovine serum medium with dimethylsulfoxide, 10, or 25 µmol/l (a) dihydroartemisinin (DHA), (b) ON-2Py, or (c) 2Py for 24, 48, and 72 h. Relative cell number was assessed by crystal violet assay ($n=4$). Results are expressed as the mean ± SD. *Significant difference from control ($P<0.05$).

Effect of dihydroartemisinin, ON-2Py, and 2Py on cell number

The cell number was initially assessed by crystal violet assay (Fig. 3). DHA had no significant effect on reducing the cell number in C4-2, LNCaP, or PC-3 cells, with the exception of one data point for C4-2 cells after 72 h of treatment under the conditions we used in this study (Fig. 3a). ON-2Py at both 10 and 25 µmol/l concentrations had a significant effect on reducing cell number for all three cell lines at the 72-h time point. This decrease in cell number was more evident in the C4-2 and LNCaP cells. ON-2Py was the most effective compound at decreasing cell number at the 10 µmol/l concentration (Fig. 3b). However, the most significant decreases in cell number were observed using 25 µmol/l 2Py, which significantly decreased all three cell lines to approximately 15% of the control cell number after 72 h (Fig. 3c). The IC_{50} values calculated for 2Py at the 48-h time point were 16.24, 28.53, 9.59, and 17.11 µmol/l for the C4-2, DU 145, LNCaP, and PC-3 cells, respectively.

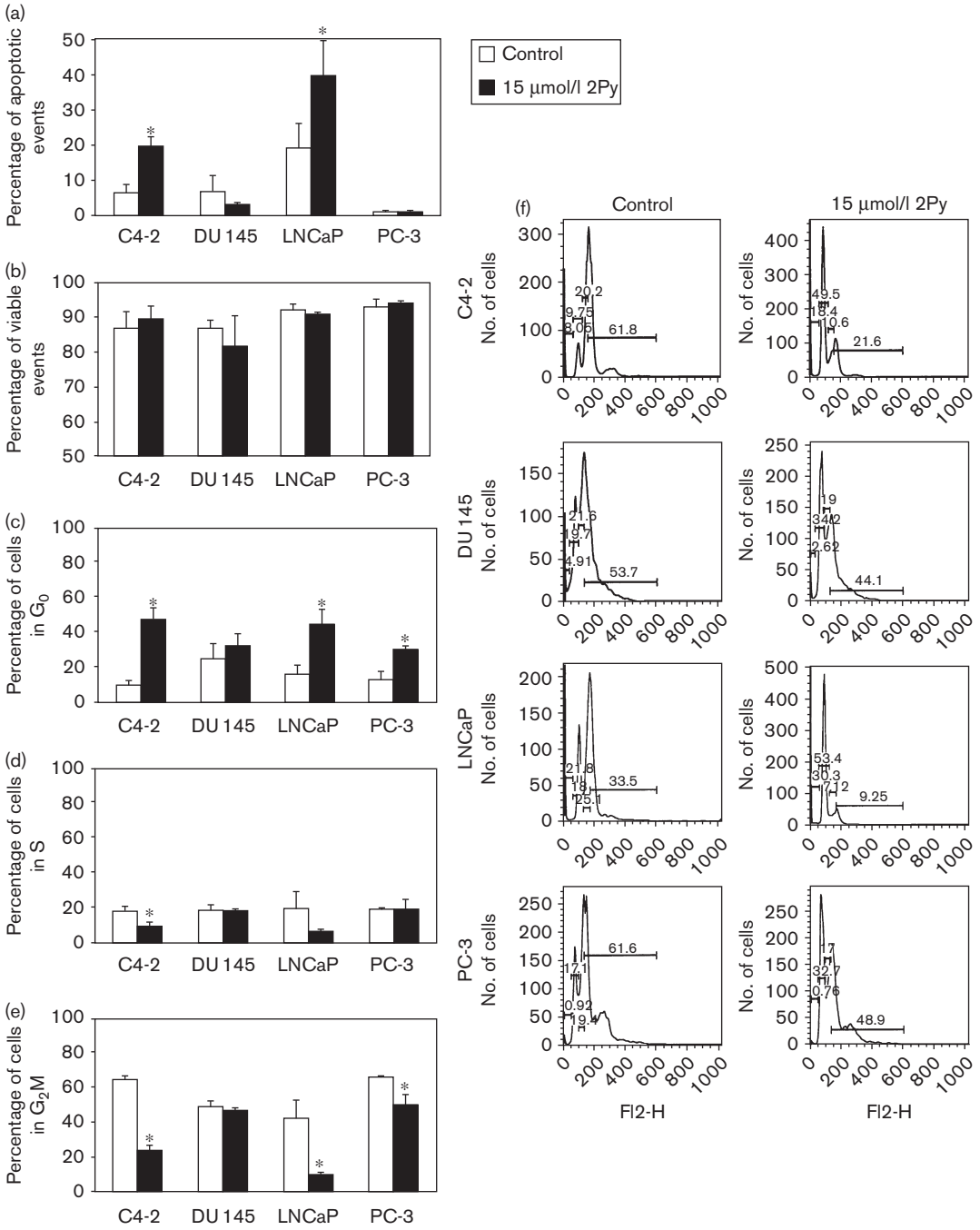
To determine whether the effects of 2Py were related to the levels of transferrin available, C4-2, DU 145, LNCaP, and PC-3 cells were treated with 5, 10, and 15 µmol/l concentrations of the artemisinin derivative with or without iron-saturated human holo-transferrin for 48 h.

The cell number was measured by an MTT assay. Although there were subtle differences in cell number in all cases, there was no significant effect of holo-transferrin on cell number. Furthermore, 2Py had a limited effect on decreasing cell number in DU 145 cells when compared with the other cell lines (data not shown).

Effect of 2Py on apoptosis and cell cycle

DU 145 and PC-3 cells had no statistically significant increase in apoptotic events with 2Py treatment; however, both C4-2 and LNCaP cells had an increase in apoptotic events in response to 15 μmol/l 2Py treatment (Fig. 4a). Membrane integrity (viability) was not altered

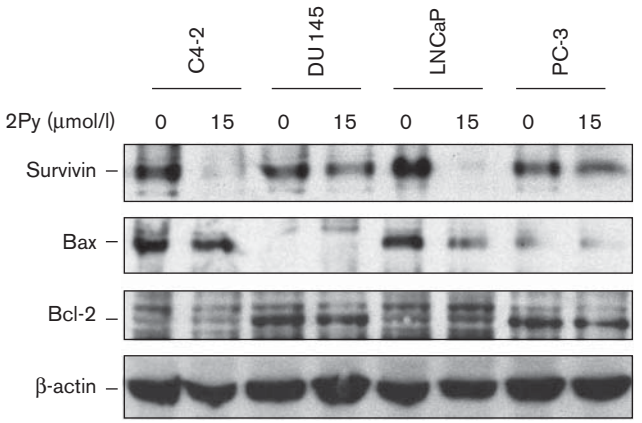
Fig. 4



Effect of 2Py on apoptosis and cell cycle in LNCaP, C4-2, PC-3, and DU 145 cells. Cells were brought to 50% confluence and then cultured in RPMI 1640 with 10% fetal bovine serum medium with dimethylsulfoxide or 15 μmol/l 2Py for 48 h. The cells were trypsinized and stained with propidium iodide and analyzed on a flow cytometer. The percentage of apoptotic events (a), cell viability (b), and the percentage of cells in G_0, G_2M and S phase of the cell cycle (c–e) were determined ($n=3$). Histograms of cell cycle distribution in control and 2Py-treated cells (f).

between doses (Fig. 4b). As the loss in cell number in the PC-3 cell line could not be attributed to apoptosis, we examined the effect of 2Py on cell cycle kinetics. 2Py (15 μmol/l) increased the percentage of cells in the G_0 phase of the cell cycle in all cell lines, and significantly increased it in the C4-2, LNCaP, and PC-3 lines (Fig. 4c). The same dose of 2Py significantly decreased the number of cells in G_2M, and S phase in the C4-2, LNCaP, and PC-3 cells, with no effect on the DU 145 cells (Fig. 4d).

Fig. 5



Effect of 2Py on survivin, Bcl-2, and Bax in LNCaP, C4-2, PC-3, and DU 145 cells. Cells were brought to 50% confluence and then cultured in RPMI 1640 with 10% fetal bovine serum medium with dimethylsulfoxide or 15 μmol/l 2Py for 48 h, cell lysates were isolated and western blotting analysis performed using antibodies to survivin ($n=4$), Bax ($n=3$), and Bcl-2 ($n=3$), with β-actin as control.

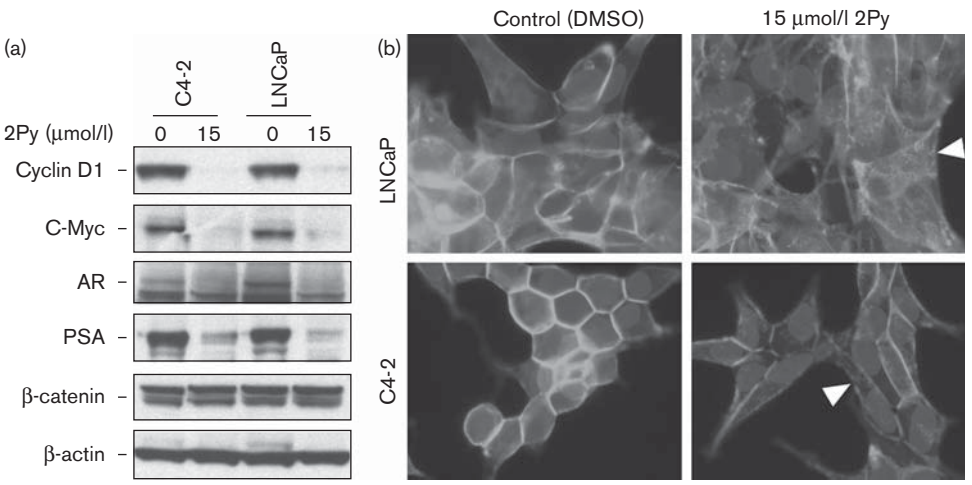
Apoptosis-associated protein expression

2Py had a limited effect on DU 145 cell number. 2Py altered cell cycle kinetics, but did not induce apoptosis in PC-3 cells whereas it induced apoptosis and altered cell cycle kinetics in C4-2 and LNCaP cells. To determine why there was a differential response to 2Py in each of the PCa lines, we examined the expression of apoptosis-associated proteins. 2Py significantly decreased survivin expression in all four PCa cell lines, with a more pronounced effect in the more sensitive C4-2 and LNCaP cells. As expected, the proapoptotic Bax expression levels were high in C4-2 and LNCaP cells and low in the DU 145 and PC-3 cell lines, whereas the anti-apoptotic protein BCL-2 levels were low in C4-2 and LNCaP cells and higher in DU 145 and PC-3 cells. We saw no significant change in Bax or BCL-2 levels after 2Py treatment (Fig. 5).

Proliferation-associated protein expression

Both cyclin D1 and c-Myc were earlier shown to have a role in regulating cell cycle kinetics in PCa [18,19]. We observed a dramatic decrease in both cyclin D1 and c-Myc expression levels in C4-2 and LNCaP cells treated with 2Py (Fig. 6a). Cyclin D1 and c-Myc can be regulated by β-catenin, so we examined the effect of 2Py on β-catenin expression in the C4-2 and LNCaP cells. 2Py did not alter β-catenin expression in both cell lines (Fig. 6a). Furthermore, using immunocytochemistry we

Fig. 6



Effect of 2Py on C-Myc, cyclin D1, androgen receptor (AR), prostate-specific antigen (PSA), and β-catenin expression in LNCaP and C4-2 cells. (a) Cells were brought to 50% confluence and then cultured in RPMI 1640 with 10% fetal bovine serum (FBS) medium with dimethylsulfoxide or 15 μmol/l 2Py for 48 h, cell lysates were isolated and western blotting analysis performed using antibodies to cyclin D1 ($n=3$), c-Myc ($n=3$), AR ($n=3$), PSA ($n=3$), and β-catenin ($n=2$) with β-actin as control ($n=3$). (b) Immunocytochemical analysis of β-catenin in LNCaP and C4-2 cells cultured in 10% FBS medium with dimethylsulfoxide (DMSO) or 15 μmol/l 2Py for 24 h. Arrow heads indicate cytoplasmic relocalization of β-catenin after 2Py treatment.

also determined that there was no change in the nuclear localization of β-catenin in the C4-2 and LNCaP cells after treatment with 2Py. There was, however, an increase in punctuate β-catenin cytoplasmic staining in both LNCaP and C4-2 cells after treatment with 2Py (Fig. 6b).

The effect of 2Py on the androgen receptor and prostate-specific antigen

The AR, when present, is known to play a major role in PCa tumor growth; furthermore, β-catenin is known to interact with and promote AR activity in PCa cells [20]. We determined the effect of 2Py on the levels of expression of the AR and the AR-regulated protein PSA in the C4-2 and LNCaP cells. 2Py decreased AR and PSA expression in both cell lines (Fig. 6a).

Discussion

Artemisinin has been used as an anti-malarial drug with limited toxicity for a number of years [3,5]. The activity of artemisinin in PCa cells is dependent on a heme-catalyzed reaction and TfR is required to maintain intracellular iron levels. We analyzed TfR expression in PCa bone and soft tissue metastases from our PCa rapid autopsy program at the University of Washington. Nearly all of the metastatic cores examined had TfR expression with some tumors expressing very high levels. The expression of TfR in the patient samples as well as the LuCaP xenografts and PCa cell lines suggested that artemisinin may have potential efficacy in this solid tumor type. The high levels of TfR expression in the PCa metastases are in contrast to TfR levels that are essentially undetectable in normal tissues [21,22].

Artemisinin has been used at high doses *in vivo* inhibiting PCa tumor cell growth and at up to 300 μmol/l *in vitro* blocking PCa cell proliferation [8]. Although high doses of artemisinin are well tolerated in patients treated for malaria, we synthesized two more potent derivatives, ON-2Py and 2Py. These compounds are based on artemisinin dimers reported by Posner *et al.* [3,12], as artemisinin dimers have shown significantly higher anti-cancer activities, compared with the corresponding monomers. To determine the efficacy of artemisinin *in vitro*, we treated PCa cell lines with lower concentrations of DHA. At lower concentrations the dimer, 2Py, was more effective than artemisinin at reducing PCa cell number *in vitro* for all the PCa cell lines tested. Furthermore, the IC_{50} values calculated for 2Py show that C4-2, LNCaP, and PC-3 cells were more responsive to 2Py than DU 145 cells. Interestingly, 2Py (15 μmol/l) was more potent than artemisinin in LNCaP cells, where it altered cell cycle kinetics and increased apoptotic events, whereas Willoughby *et al.* [8] showed that a higher dose of artemisinin (300 μmol/l) altered cell cycle kinetics, but did not increase apoptotic events in LNCaP cells.

The addition of holo-transferrin to cells can increase the effect of DHA *in vitro* by increasing intracellular levels of holo-transferrin [Fe(III)] in the cells [23]. The addition of holo-transferrin in combination with 2Py had no additive or synergistic effect on the PCa cells. We speculate that 2Py does not act solely through free intracellular Fe(II) or that the levels of transferrin present in the PCa cells is limited, thereby restricting the availability of intracellular holo-transferrin [Fe(III)] [2]. PCa cells are known to accumulate a large amount of iron [24], and the level of intracellular labile iron in those cells may not be affected by added holo-transferrin. Furthermore, Efferth *et al.* [25] reported that the modulation effects of added iron on the efficacy of artesunate vary significantly among different cell lines.

Artemisinin-derived compounds have been shown to induce apoptosis through a mitochondrial-mediated pathway. The apoptotic effect of 2Py on the C4-2 and LNCaP cells may be because of their predisposition to apoptosis through a mitochondrial-mediated pathway, with both cell lines having low levels of the anti-apoptotic protein, Bcl-2, and higher levels of the proapoptotic protein, Bax. Survivin is undetectable in terminally differentiated adult tissues and is a key regulator of cell survival and cell cycle progression and has been described as a molecular target in PCa [26,27]. Interestingly, treatment with 2Py decreased the anti-apoptotic survivin protein levels in all of the PCa cell lines.

Willoughby *et al.* [8] have described the molecular actions of artemisinin as disrupting Sp1 interactions with the cyclin-dependent kinase-4 (CDK4) promoter, thereby inhibiting CDK4 expression and causing cell cycle arrest. The enzymes that regulate G_1 to S phase transition are CDK2, CDK4, and CDK6. CDK4 and CDK6 complex with D-type cyclins, phosphorylating the retinoblastoma protein promoting proliferation [28]. Suzuki *et al.* [29] have shown that survivin interacts with CDK4, leading to CDK2/cyclin E activation and retinoblastoma protein phosphorylation. As a result of the formation of the survivin/CDK4 complex, the CDK inhibitor p21 can be released from its complex with CDK4 and interact with mitochondrial procaspase 3 to suppress cell death. Therefore, the loss of survivin, cyclin D1, and possibly CDK4 could considerably decrease proliferation and promote a proapoptotic phenotype in PCa cells.

C-Myc is a growth-regulatory transcription factor strongly implicated in prostate carcinogenesis. It is often amplified in advanced PCa and is involved in androgen-independent growth of PCa [30,31]. Furthermore, the overexpression of c-Myc and cyclin D1, as well as phosphorylating the retinoblastoma protein, promotes G_1–S transition and proliferation. Therefore, it is likely that the loss of c-Myc protein in 2Py-treated cells also has a role in decreasing proliferation in PCa cells.

As cyclin D1 and c-Myc are downstream targets of β-catenin in PCa, we examined protein levels and nuclear localization of β-catenin in the LNCaP and C4-2 cell lines after 2Py treatment. β-catenin protein levels and nuclear localization were not altered significantly with 2Py treatment, suggesting that the loss of cyclin D1 and c-Myc expression is not a result of changes in β-catenin expression levels or nuclear localization [32]. We did, however, observe an increase in punctuate cytoplasmic staining after 2Py treatment in the C4-2 and LNCaP cells. This loss of membrane-associated β-catenin and increase in punctuate cytoplasmic staining may relate to the initial loss of cell–cell contact in the early stages of anoikis, before apoptosis occurs in these two cell lines.

The loss of AR and PSA expression in the C4-2 and LNCaP cells is a novel finding for an artemisinin derivative. PSA expression is androgen-dependent, and the promoter of the PSA gene is regulated by AR [33]. It is speculated that 2Py causes a decline in AR expression that subsequently results in a decrease in PSA levels. AR promotes both androgen-dependent and castration-resistant PCa tumor growth; therefore, the deleterious effect of 2Py on AR and PSA expression in the AR-expressing lines is a potentially significant finding and requires further investigation.

In summary, we have determined that at lower concentrations 2Py is more effective than artemisinin at causing cell cycle arrest and apoptosis in PCa cells *in vitro*. Furthermore, the loss of survivin and AR in response to 2Py treatment is a significant finding and warrants further study. The continued development of more analogs of artemisinin may result in a more potent compound with greater activity, leading to the development of more effective therapies for PCa.

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